6-2008

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Secondary Metabolism Inducing Treatments During In Vitro Development of Turmeric (*Curcuma longa* L.) Rhizomes

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Key words: antioxidant, chelating, phenolics, curcumin, DPPH*, methyl jasmonate, phenylalanine, chitosan

Abstract

Turmeric (*Curcuma longa* L.) plants were grown *in vitro* for 17 or 22 weeks as a fed-batch culture in 2.5 L vessels that yielded 39-43 and 62-70g rhizome FW per vessel (95% confidence interval) for the 17 and 22 week experiments, respectively. MS liquid medium was maintained at 6 % sucrose through media addition twice during the experiment. Various methods were employed in attempts to upregulate secondary metabolism, antioxidant and total phenolic assays were employed to characterize phytochemical activity. A first experiment exposed four clones to phenylalanine and/or methyl jasmonate (MeJa) from week 12 to 17. In a second experiment on one clone, short-term exposure (1.5 weeks) to proline, a natural proline-rich fish extract, MeJa, and chitosan began during the 20^{th} week of culture. This experiment also included a nitrogen stress treatment (weeks 16-22). The 5 week phenylalanine and MeJa treatments lowered biomass accumulation and antioxidant capacity of the tissue. The magnitude of antioxidant depression was dependent on genotype, and within each genotype, the degree of depression was similar for phenylalanine and MeJa, alone and in combination. In the second experiment, only the low nitrogen treatment yielded an increase in phenolic content to 4.7% of dry weight compared to untreated micro-rhizomes (4.1% of dry weight). Nitrogen stressed plants also had less leaf growth, but rhizome mass was unaffected and averaged 63 g FW per vessel. None of the short-term treatments had a significant effect on biomass, antioxidant capacity, or phenolic content. None of the treatments significantly affected radical scavenging although the

low nitrogen treatment might have improved this activity ($p = 0.1207$). Results indicated that plants grown in a high nitrogen MS media were not responsive to elicitation.

Abbreviations: Benzyladenine – BA; Methyl Jasmonate – MeJa; Dry Weight – DW; Fresh Weight – FW; Relative Dry Weight (DW/FW) – RDW; Murashige and Skoog Media (Murashige and Skoog, 1962) – MS

Introduction

Turmeric (*Curcuma longa* L.) has been used in traditional Indian medicine (Ayurvedic Medicine) for hundreds of years and has recently gained increased attention due to its significant medicinal potential. Commercially, it is used as a dye, spice, and industrial starch. It is also a major ingredient for making curry powder, which is commonly consumed in the countries of southern and eastern Asia and is a major part of most curries.

Much work has been carried out on the antioxidant and related anti-cancer activities of compounds extracted from turmeric rhizomes. The curcuminoids are major antioxidative compounds and largest group of phenolic compounds found in turmeric. Many other compounds, including volatile essential oils, also possess antioxidant properties (9). These compounds include: γ-terpinene, ascorbic-acid, beta-carotene, beta-sitosterol, caffeic-acid, campestrol, camphene, dehydrocurdione, eugenol, p-coumaric-acid, protocatechuic-acid, stigmasterol, syringic-acid, turmerin, turmeronol-a, turmeronol-b and vanillic acid (9).

Many attempts have been made to use plant cell culture for production of plant secondary metabolites, but by far, most of these attempts have not been cost effective and only four commercially viable systems have been reported (2). Factors that were blamed for this failure

included lack of storage cells for accumulation of secondary metabolites, low yield, and a high cost of equipment. Hairy root culture employs differentiated organs and often produces better yields than undifferentiated cells. We have used whole plant culture in a simple mechanical system for production of differentiated microrhizomes with potential for accumulation and storage of secondary metabolites (1, 6).

A wide variety of biochemical strategies have been utilized to enhance *in vitro* production of secondary metabolites. Plant defense hormones such as jasmonates have been shown to induce secondary metabolite production (14, 16, 11, 33). Jasmonic acid was also found to stimulate storage organ formation in garlic with a 10 μM solution being optimal (20). Phenylalanine ammonia lyase (PAL) is the first step in the shikimic acid acid pathway and a key enzyme that links primary metabolism to secondary metabolism by serving as a catalyst in the deamination of phenylalanine, was found to be activated by exposure to methyl jasmonate (27). Phenylalanine is a precursor for most phenolic compounds in plants and has been successfully used to induce metabolite production in vitro in many different plant systems (17, 12, 25).

Fungal elicitation also works as an inducer of secondary metabolism. Exposure of basil to chitosan (a fragment of fungal cell wall polysaccharides) caused significant increases in medicinal compound production (19). Chitosan hydrolysates increased paclitaxel production in cell cultures of *Taxus canadensis* (23).

Exposure of plant materials to the amino acid L-proline has been shown to increase activity of the pentose phosphate pathway and leads to greater activity from the shikimic acid pathway and phenyl propanoid pathways. Proline and one of its analogs, hydroxyproline, have been shown to cause increases in phenolic and rosmarinic acid contents of thyme (22) oregano (32) and pea (11).

Lowering the concentrations of inorganic nitrogen has been shown to lead to increased metabolite production. Flavonol accumulation was promoted in plants that were nitrogen deficient (28). Nitrogen deficiency led to increased levels of phenolics and increased activity of phenylalanine ammonia lyase (21). In tobacco, nitrogen deficiency led to a shift from nicotine production to the synthesis of larger carbon rich metabolites (chlorogenic acid and rutin) (13). Lowering nitrogen concentration from that contained in MS media led to increases solasodine production from *Solanum khasianum* (18). The Murashige and Skoog (24) tissue culture medium (rich in inorganic nitrogen – 20 mM ammonium and 40 mM nitrate) is used most commonly for herbaceous plants (15). This includes all cited studies with turmeric (3, and references therein).

In our current study, the antioxidant potential and phenolic content of in vitro turmeric microrhizomes grown under different conditions was evaluated. These two activities were selected as a way to effectively characterize the quality and medicinal potential of the tissue produced. First, the effects of MeJa and the phenolic precursor, phenylalanine, alone and in combination, were measured. The complex turmeric extracts that contain many antioxidant constituents were examined using two assays. The DPPH* free radical scavenging assay was used to determine a “primary” radical scavenging potential, and the ferrous iron chelating assay determined a “secondary” radical scavenging potential that demonstrates the extract’s ability to chelate metals in biological systems – preventing reactive oxygen species generation. A second experiment with one selected clone quantified the effects of MeJa (a defense hormone), chitosan (a fungal elicitor), proline (a pentose phosphate pathway upregulator), Gropro (a complex natural extract rich in proline), and nutrient stress (low nitrogen) on antioxidants and total phenolics. Antioxidants were measured (as above) with the DPPH* free radical scavenging assay (31). Total phenolics (8) were measured using the total phenolics assay.

Materials and Methods

Plant Materials

Four accessions of turmeric, *Curcuma longa* L., (L22-5, L35-1, L43-4, and L50-3) were obtained from the University of Arizona Southwest Center for Natural Products Research and Commercialization. Plant tissue culture stocks (stage one) were prepared for this experiment as described by Cousins and Adelberg (5).

Chemicals

Ferrozine, ferrous chloride ($FeCl_2$), tris-HCl, 2,2-diphenyl-1-picrylhydrazyl (DPPH*), Folin-Ciocalteau Reagent, sodium carbonate (Na_2CO_3), and proline were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). 2,6-di-tert-butyl-4-methylphenol (BHT) was purchased from ACROS (NJ). Methanol was obtained from Fisher Scientific (Suwanee, GA). Ethylenediaminetetraacetic acid (EDTA) was purchased from JT Baker Chemical Co. (Phillipsberg, NJ). Methyl jasmonate and L-phenylalanine were purchased from PhytoTechnology Laboratories (Shawnee Mission, KS). Gropro and chitosan were obtained from Dr. Kalidas Shetty at the University of Massachusetts – Amherst.

Phenylalanine and MeJa

After stock preparation, subcultured rhizomes were placed in flasks at a rate of 3-4 buds per jar with 30 ml of media per jar. Following the 5 week period in the jars, the plants were moved to a thin-film rocker system as reported in Adelberg and Cousins (1). The rocker vessels have approximately 2.5 L volume with 325 cm^2 growing surface and are agitated through

periodic vessel tilting. The media used was modified MS media of the same formulation as used in the shake flask culture system. Twelve to eighteen buds were placed in each box along with 200 ml of media. Additional media (100 ml) was added to the vessels twice during the experiment, once at 12 weeks and again at 15 weeks. Two boxes from each clone were given media additions of the following types: same as above, same as above plus 10 µM MeJa, same as above plus 6 mM phenylalanine, and same as above plus 10 µM MeJa and 6 mM phenylalanine. There were two replicates of each type of box in the 4 clones x 2 MeJa levels x 2 phenylalanine levels factorial. Treatment concentrations were selected in accordance with a preliminary experiment conducted by the authors in the case of MeJa and through a perusal of the literature in the case of phenylalanine. A summary of methods can be found in Figure 1.

Proline, Gropro, Chitosan, MeJa, and Nitrogen Deficiency

Plant material was similar to that used in experiment one, but only clone L50-3 was utilized. L50-3 was selected because its metabolic response in the previous experiment was typical of the majority of the clones. Rhizomes were placed in boxes at a rate of 14 buds per box with 200 ml of media. An additional 200 ml of media with the same formulation as above was added during week 10. Another media addition was made during week 16. The low nitrogen treatment began during week 16 when nitrogen free MS medium was added to three vessels. Vessels for other treatments were supplemented with MS media as in week 10. During week 21, a process of known addition was used to estimate the media volume in each vessel. Sucrose concentrations of media in vessels were measured on a refractometer (Model # N-10E – ATAGO, U.S.A., Inc., Bellevue, WA). Ten ml of a 20 % sucrose MS media was added to all boxes except those assigned to the low nitrogen treatment, and nitrogen free media with 20 % sucrose

concentration was given to the low nitrogen treatment. Using the volume estimates, 2 ml treatment solutions were prepared so as to create specific vessel media concentrations of the remaining treatments. Proline (2 mM), gropro (natural source of proline – 5 ml l^{-1}), chitosan (200 mg l^{-1}), MeJa (10 μM) were applied through the addition of 2 ml solution in distilled deionized H_2O. Proline, gropro, and chitosan treatment concentration levels were selected with the assistance of Dr. Kalidas Shetty (University of Massachusetts, Amherst), based on successful in vitro manipulation of secondary metabolism in Lamiaceae. A summary of methods can be found in Figure 1.

Tissue harvesting and processing

Plants in the first experiment were harvested after 17 weeks while plants in the second experiment were harvested at 22 weeks. Media volume, concentration of sucrose remaining in the media, and numbers of buds were tabulated. Elongated leaves, rhizomes, and roots were separated and massed fresh. Portions of tissue were dried to calculate DW percentages. Twenty g portions of rhizome tissue were separated from the bulk and frozen separately for use in fresh extractions. All rhizome tissue was stored in the dark at -20 ºC prior to extraction.

Extractions, processing, and storage

The 20 g tissue samples were thawed, shredded, and placed in cellulose extraction thimbles. The thimbles were then inserted into a soxhlet apparatus equipped with a condenser. Methanol in the amount of 250 ml was placed in a 500 ml round bottomed flask attached to the base of the soxhlet. The mantle was set to 40 % of capacity. Each extraction was allowed to continue for 12 h. In the first experiment, extracts were allowed to cool to room temperature and

gravity filtered through two 20 g portions of sodium sulfate yielding a final extract volume of approximately 200 ml. In the second experiment, the volume of these extracts was standardized to 230 ml prior to a single filtration through a 20 g portion of sodium sulfate. A 50 ml aliquot of each extract was concentrated to 20 ml using a vacuum rotary evaporator. In the case of the first experiment, each of the concentrated extract samples was serially diluted by half until 8 samples of differing concentrations were available for use in the assays to follow. Concentrations ranged from approximately 26 g l^{-1} to 0.2 g l^{-1}. In the second experiment, extract samples were serially diluted by half until 7 samples were available for use in assays. Concentrations ranged from approximately 20 g l^{-1} to 0.2 g l^{-1}. All concentrations were calculated on a rhizome DW basis. Extracts were stored in the absence of light at -5 ºC until assays could be preformed.

Free radical scavenging

Free radical scavenging effect was determined using the free radical generator DPPH* (2,2-diphenyl-1-picrylhydrazyl) by a similar method to that used by Yamaguchi et al. (31). Two hundred µl aliquots of the serially diluted extract samples were placed in 12 x 75 mm culture tubes with 800 µl of Tris-HCl pH 7.4. One thousand µl of 500 µM DPPH* solution were added to the resulting mixture. The reaction mixture was thoroughly mixed using a vortex and placed in the dark for 20 minutes. After the dark treatment, absorbance was measured via spectrophotometer at 517 nm on a Spectronic 20 Genesys™ spectrophotometer (Fisher Scientific, Fairlawn, NJ). Scavenging activity was calculated via Equation 1.

$$\text{Scavenging Activity (\%)} = \left(1 - \frac{\text{Absorbance of Sample at 517 nm}}{\text{Absorbance of Control at 517 nm}}\right) \times 100 \quad \text{(Equation 1)}$$

Fe^{2+} chelating effect

Ferrous iron chelating effects were measured using the method explained by Decker and Welch (8) with some modification. This assay used the formation of a ferrous iron ferrozine complex to spectophotometrically monitor the iron chelating ability of the plant extracts observed in this experiment. Eight hundred µl aliquots of the serially diluted extracts were placed in 12 x 75 mm culture tubes with 200 µl of 0.2 mM $FeCl_2$ and 1 mM Ferrozine. The mixtures were then thoroughly shaken and allowed to stand for 10 min at room temperature. The absorbances were then measured at 562 nm on a Spectronic 20 Genesys™ spectrophotometer (Fisher Scientific, Fairlawn, NJ), and the chelating effects were determined via Equation 2.

$$\text{Chelating Effect (\%)} = \left(1 - \frac{\text{Absorbance of Sample at 562 nm}}{\text{Absorbance of Control at 562 nm}}\right) \times 100 \qquad \text{(Equation 2)}$$

Total phenolics

The concentration of total phenolic compounds in the extracts was quantified as equivalent amounts of gallic acid. A 200 µl aliquot of rhizome extract was placed in a 16 x 100 mm culture tube followed by 800 µl distilled deionized H_2O, 4 ml saturated Na_2CO_3 solution and 5 ml of 0.2 N Folin-Ciocalteau reagent. This mixture was vortexed and allowed to incubate for 2 hours at room temperature. Solutions were placed on ice before being read on a Spectronic 20 Genesys ™ spectrophotometer (Fisher Scientific, Fairlawn, NJ) at 765 nm. Results from assays were compared with a gallic acid standard curve and were expressed in µg gallic acid per mg rhizome DW.

Experimental design and analysis

In experiment one, biomass comparisons were made using ANOVA α=0.05. Antioxidant activities of rhizome tissue from the four clones in four treatment conditions were compared in a

factorial design. The untreated clones served as controls and were compared with each treatment within clone. DPPH* free radical scavenging and iron chelating curves were established by fitting the data to a hyperbolic tangent function (6). Estimates of the EC_{50}s (extract concentration that gives 50% of maximum effect) were obtained using the maximum likelihood method, and Wald's *z*-test was used for all comparisons (4). In experiment two, biomass comparisons were conducted as above. EC_{50} values were calculated and compared using ANOVA α=0.05, and a Dunnett's test was used to compare treatments with the control.

Results

At harvest, large amounts of tissue tightly filled the 2.5 L rocker vessels (Figure 2). Rhizomes were well differentiated and contained the bright yellow coloring characteristic of curcuminoids (Figure 3). Noteworthy plant organ biomass yield was demonstrated in all treatments. Rhizome FW of 39-43 g per vessel (95% confidence interval) grew over a 17-week period (Table 1). Each 2.5 L vessel occupies 325 cm^2 of bench space, making in vitro growth of rhizomes an efficient process with regard to space and time. Leaf blades, rhizomes and roots, all exuded a pleasant odor indicating the presence of volatile compounds.

Phenylalanine and MeJa

Turmeric plants treated with MeJa and phenylalanine, alone and in combination, yielded decreased rhizome FW accumulation in vitro (Table 1). MeJa and phenylalanine had negative effects of similar magnitude, and their combined effect was also similar to the effect of either treatment alone. The interaction between MeJa and phenylalanine was significant. MeJa and phenylalanine effects were only visible in the absence of the other treatment. For example, MeJa

had a negative effect on biomass accumulation in the absence of phenylalanine, but in the presence of phenylalanine, there was no discernable MeJa effect.

Rhizome RDW (ratio of DW/FW) was not affected by MeJa or phenylalanine, but varied from 9 -11% dependent on clone. Whole plant FW was negatively impacted by phenylalanine, MeJa and phenylalanine and MeJa (data not shown). Whole plant FW of varied by clone.

In all four clones, phenylalanine and MeJa alone and in combination decreased the antioxidant potential of the rhizome extracts (Table 2). Both MeJa and phenylalanine reduced antioxidant potentials by a similar amount, and their combined effect was similar to the effect of either chemical alone. This was consistent for all 4 clones even though there were differences in the size of the response from clone to clone. For example, in the clone with the strongest antioxidant potential (L 22-5), MeJa and phenylalanine increased EC_{50}s by factors of 7.8 and 7.4 respectively. In combination, the two treatments caused the EC_{50}s to increase by a factor of 7. In the clone with the weakest antioxidant potential (L 35-1), phenylalanine or MeJa increased EC_{50}s by a factor of 2.7. In combination, the EC_{50} was increased by a factor of 2.5.

Iron chelation data was inconsistent across clones for the treatments (data not shown). In hindsight, some EDTA from the medium might have been incorporated into the rhizome where it was extracted and caused unpredictable effects on the chelation assay. Despite this realization, nothing in the chelation assay data caused us to doubt the general conclusion that MeJa and phenylalanine did not enhance the antioxidant potential of the turmeric microrhizomes.

Proline, Gropro, Chitosan, MeJa, and Nitrogen Deficiency

We selected the clone L 50-3 from the first experiment as its responses were typical with regard to MeJa and phenylalanine where treatments caused significant reductions in antioxidant

potential. Unlike the longer term experiment, the short-term treatment exposure in this second experiment did not alter rhizome biomass, and all the treatments except for the low nitrogen treatment had the same mass as the control. Rhizome FW of 62-70 g per vessel (95% confidence interval) was produced in this experiment over a 22-week period. The low nitrogen treatment lowered shoot FW and DW accumulation but had no effect on rhizome biomass (Figure 4). The nitrogen deficient plants produced more phenolics (4.7% of DW) than the control (4.1% of DW)(Table 3). No other treatment had a significant effect on phenolic production. There was also a difference between the effect of proline and the effect of Gropro in this experiment.

None of the treatments altered the ability of extracts to scavenge the DPPH* radical to a significant degree (Table 3). EC_{50}s of extracts from tissue treated with chitosan, Gropro, nitrogen deficient media, MeJa, and proline were not significantly different from the control. Low nitrogen might have had the effect of decreasing the EC_{50} or increasing scavenging efficacy (low nitrogen vs. control; $p < 0.1207$). Three replications were utilized due to economy of working in 2.5L vessels over long time frames. Larger numbers of replications may have yielded statistical significance.

Discussion

As a part of any plan to enhance secondary metabolite production in a plant or organ culture system, two factors must be considered – biomass production and metabolite concentration in tissue. Biomass and microrhizome development were promoted by the plant growth regulator (PGR) benzyladenine, correlated to sugar supplied in the medium, and may be maintained for several months by repeated additions of sugar containing medium (5). MeJa did not increase the biomass of plants or rhizomes.

Phenylalanine and MeJa

The results clearly indicated negative effects on biomass and secondary metabolism from both precursor and hormone treatments. These negative effects have several possible explanations. One likely explanation is that phenylalanine and MeJa altered primary metabolism and plant growth. This is most likely because the magnitudes of effects were similar for phenylalanine, MeJa, and the combined treatments. This suggests that the mechanism by which inhibition occurred was similar for the treatment conditions. Phenylalanine or MeJa created an imbalanced condition that interfered with growth. The reduction in primary metabolism coincided with reduced secondary metabolism. It is unlikely that different mechanisms would cause effects of the same magnitude in the same direction and not be additive.

A few other possible causes may be considered. Phenylalanine may have caused feedback inhibition in the shikimic acid pathway. Metabolites may have been induced that were not detected by our assays; this enhancement would have channeled carbon away from the metabolites we measured. Induction may have occurred in the short-term. Lastly, positive effects on secondary metabolism could have been overshadowed by primary metabolism depression from the long period of exposure to the compounds. Experiment two contained shorter time periods, and metabolic precursors besides phenylalanine, to circumvent these types of problems.

Proline, Gropro, Chitosan, MeJa, and Nitrogen Deficiency

Results for the second experiment were quite different from the first. Here, no treatments affected rhizome biomass and only one altered shoot biomass. The ten day treatment period probably did not allow differences in plant growth to become apparent as observed in the first experiment. MeJa reduced antioxidant potential in the first experiment but had no effect on total

phenolics in the second experiment. Since these two activities are often closely related, these results seem paradoxical. This apparent contradiction is the result of different treatment periods for each experiment. Ten days in the second experiment did not cause a negative effect while 5 weeks in the first experiment did.

Most important, however, is the finding that nitrogen-stressed plants produced the same amount of rhizome mass while synthesizing higher levels of phenolics. This constitutes an increase in tissue quality. Additionally, the nearly significant increase in DPPH* radical scavenging serves to support the finding of increased total phenolic content. No other treatment had a positive effect on tissue quality.

Reports from other groups with in vitro turmeric microrhizomes do not contain information on phytochemical activities. In our current work total phenolics were raised to 4.7% of rhizome mass by nitrogen stress. Field grown plants contain 2-6% curcuminoid phenolics after 1 or 2 years in the field.

In whole plant and organ culture, both concentration of active compounds and biomass must be considered in medicinal plant production. Turmeric is typically grown in nitrogen-rich MS medium to promote maximum biomass production. Our attempts at induction of secondary metabolism in the first experiment may have been less effective in this nutrient-rich environment. As indicated by the second experiment, it may not be possible to successfully upregulate metabolite production thus increasing tissue quality in turmeric in an environment containing luxuriant amounts of nitrogen. Sugar use has been related to rhizome biomass through primary metabolism (6). The next step, as indicated by this research, is optimization of carbon use in a low nitrogen environment followed by attempts to upregulate secondary metabolism using growth regulators or precursors in an optimized carbon/nitrogen system. This methodology is a

powerful tool for better understanding of the quality tissue production processes and a gateway to construction of a model from sugar use and nutrient status to metabolite production.

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Table 1. Rhizome FW (g per vessel) for each clone treated with neither phenylalanine nor MeJa, Phenylalanine only, MeJa only, and both phenylalanine and MeJa.

Clone[1]	Control[2]	Phe	MeJa	Both
		(6 mM)	(10 µM)	
L22-5	42.000	34.790*	36.950*	34.305*
L35-1	53.865	35.610*	37.750*	40.700*
L43-4	44.540	38.270*	42.030*	42.170*
L50-3	50.500	39.420*	36.730*	42.110*
Average	47.726	37.023	38.365	39.821

[1]No clonal effect was observed.

[2]Neither Phenylalanine nor MeJa.

*Denotes value that is significantly different from the control when α=0.05.

Table 2. DPPH* free radical scavenging of methanolic rhizome extracts from four *in vitro* grown clones of turmeric grown in media containing hormone and/or precursor.

Clone	MeJa	Phe	EC_{50} (gl^{-1})	Variance	Difference[1]	
L22-5	no	no	1.046	0.00816		
L22-5	yes	no	8.190	0.02848	-7.143	***
L22-5	no	yes	7.763	0.18488	-6.717	***
L22-5	yes	yes	7.357	0.13597	-6.311	***
L35-1	no	no	1.816	0.02122		
L35-1	yes	no	4.946	0.04543	-3.131	***
L35-1	no	yes	4.883	0.03317	-3.068	***
L35-1	yes	yes	4.667	0.00216	-2.851	***
L43-4	no	no	1.551	0.00110		
L43-4	yes	no	3.995	0.00759	-2.444	***
L43-4	no	yes	5.162	0.02815	-3.611	***
L43-4	yes	yes	4.289	0.02044	-2.738	***
L50-3	no	no	1.151	0.00239		
L50-3	yes	no	5.057	0.03226	-3.906	***
L50-3	no	yes	4.980	0.04107	-3.829	***
L50-3	yes	yes	5.140	0.06707	-3.989	***

[1]Difference= control-treated

*, **, and *** indicate differences significant at 0.10, 0.05, and 0.01 respectively as determined by Wald's *z*-test.

Table 3. DPPH* free radical scavenging and total phenolics for in vitro grown turmeric clone L50-3 grown in normal media compared to turmeric treatments designed to induce secondary metabolite production.

Treatment	DPPH*	Variance	Difference[1]	
	EC_{50} (gl^{-1})			
Control	4.075	0.09533		
Chitosan	4.588	0.31665	-0.512	-
GroPro	4.070	0.31884	0.006	-
Low Nitrogen	3.399	0.01396	0.676	†
MeJa	3.860	0.18952	0.216	-
Proline	4.364	0.54041	-0.289	-
Treatment	Total Phenolics	Variance	Difference[1]	
	(µg GA/mg DW)			
Control	41.008	8.86814		
Chitosan	40.111	6.76497	0.898	-
GroPro	44.005	8.68599	-2.997	-
Low Nitrogen	46.888	3.05680	-5.880	***
MeJa	43.170	0.53329	-2..162	-
Proline	38.898	3.21886	2.110	-

[1]Difference= control-treated

*, **, and *** indicate differences significant at 0.10, 0.05, and 0.01 respectively as determined by Dunnett's test.

† indicates difference that would be significant at 0.15 (p value = 0.1207).

Figure 1. Methods overview flowchart including major events over the course of the experiment. Where applicable the week number in which the event occurred is given in parenthesis following the event title.

Figure 2. Tissue as viewed through the back of the vessel on the day of harvest (a), and tissue removed from a single box that has been separated by plant organ (leaf, root, and rhizome) (b).

Figure 3. Bisected turmeric rhizome shows yellow coloring of curcuminoids (a). Large intact rhizomes displaying yellow coloring and club shaped morphology (b).

Figure 4. Comparison of nitrogen stressed plants (Right) with plants from a representative control vessel (Left).

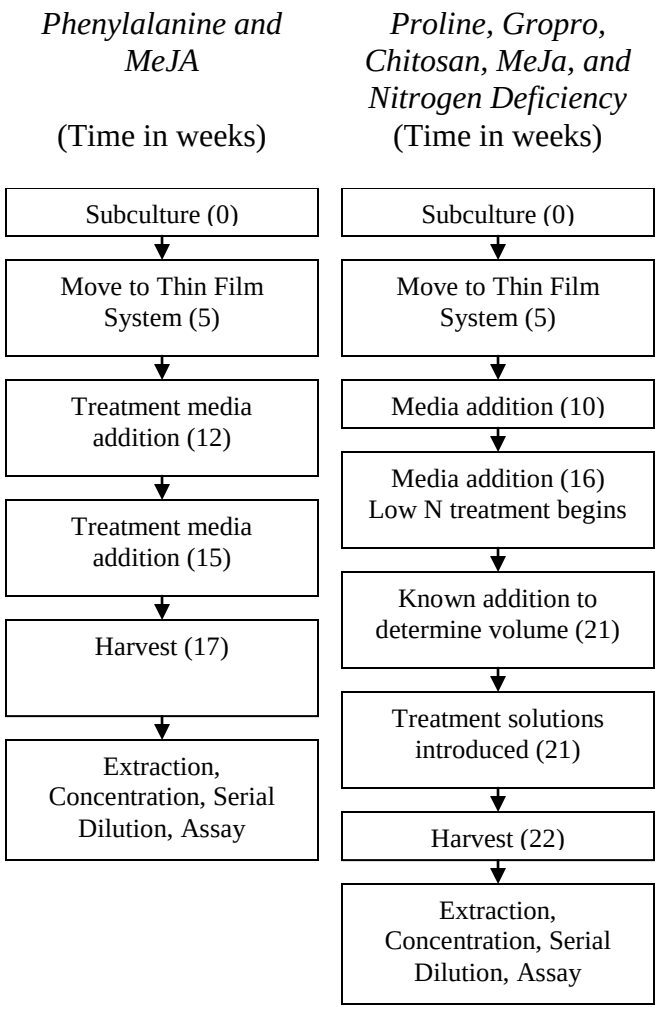
Phenylalanine and MeJA
(Time in weeks)
Subculture (0)
Move to Thin Film System (5)
Treatment media addition (12)
Treatment media addition (15)
Harvest (17)
Extraction, Concentration, Serial Dilution, Assay
Proline, Gropro, Chitosan, MeJa, and Nitrogen Deficiency
(Time in weeks)
Subculture (0)
Move to Thin Film System (5)
Media addition (10)
Media addition (16) Low N treatment begins
Known addition to determine volume (21)
Treatment solutions introduced (21)
Harvest (22)
Extraction, Concentration, Serial Dilution, Assay

Figure 1

Figure 2

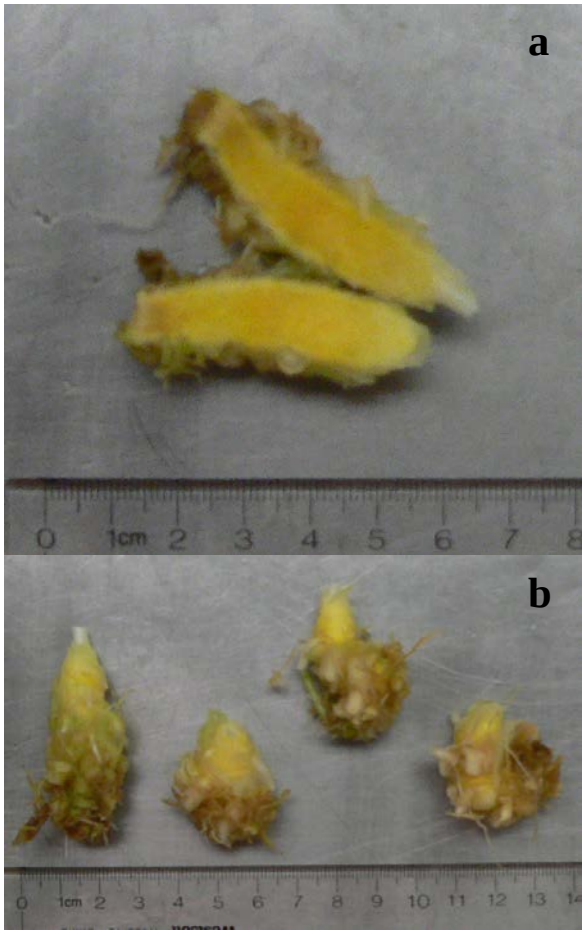

Figure 3

Figure 4